

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12P 21/06, A61K 37/00, 37/02</b> <b>A61K 37/24</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/05800</b> <b>(43) International Publication Date:</b> 17 March 1994 (17.03.94)
<b>(21) International Application Number:</b> PCT/US93/08325 <b>(22) International Filing Date:</b> 2 September 1993 (02.09.93)  <b>(30) Priority data:</b> 07/939,954 3 September 1992 (03.09.92) US  <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612-3550 (US).  <b>(72) Inventors:</b> HARLAND, Richard, M. ; 1169 Miller Avenue, Berkeley, CA 94708 (US). SMITH, William, C. ; 5918 Genoa Street, Apt. #3, Oakland, CA 94608 (US).		<b>(74) Agents:</b> SIEBERT, J., Suzanne et al.; Majestic, Parsons, Siebert & Hsue, Four Embarcadero Center, Suite 1450, San Francisco, CA 94111 (US).  <b>(81) Designated States:</b> AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, PT, RO, RU, SD, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS  <b>(57) Abstract</b>  A novel and soluble polypeptide that is highly conserved among vertebrates has been discovered, which is designated "noggin". The properties of noggin include dorsal growth inducing activity. Noggin and activin act synergistically to produce dorsal mesoderm. Noggin's cDNA has been cloned. Several expression systems have been successfully used to express biologically active noggin. Amino acid sequences representing completely conserved portions as between frog noggin and mouse noggin are: QMWLWSQTFCPVLY (SEQ ID NO: 3); RFWPRYVKVGSC (SEQ ID NO: 4); SKRSCSVPEGMVCK (SEQ ID NO: 5); LRWRCQRR (SEQ ID NO: 6); and, ISECKCSC (SEQ ID NO: 7).		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

DORSAL TISSUE AFFECTING FACTOR  
AND COMPOSITIONS

5    Field of the Invention

          The invention generally relates to growth factors and neurotrophic factors, and more particularly to a soluble growth factor with dorsal growth inducing activity, to complexes including the factor, and to DNA  
10    or RNA coding sequences for the factor.

          This invention was made with government support under Grant Contract No. R01-GM-42341, awarded by the National Institutes of Health. The government has certain rights in this invention.

15    Background of the Invention

          Growth factors are substances, such as polypeptide hormones, which affect the growth of defined populations of animal cells *in vivo* or *in vitro*, but which are not nutrient substances. Proteins involved in  
20    the growth and differentiation of tissues may promote or inhibit growth, and promote or inhibit differentiation, and thus the general term "growth factor" includes cytokines and trophic factors. Among growth, or neurotrophic, factors presently known are those that can be  
25    classified into the insulin family [insulin, insulin-like growth factors (e.g., IGF-I, IGF-II), mammary stimulating factor (MSF), and nerve growth factor (NGF)]; those classified into the epidermal growth factor family [epidermal growth factor (EGF) and  
30    transforming growth factors (TGF $\alpha$ , TGF $\beta$ , TGF $\gamma$ )]; those

classified into the platelet-derived growth factor family [platelet-derived growth factor (PDGF), osteosarcoma-derived growth factor (ODGF), and fibroblast growth factor (FGF)]; the neurotrophins [nerve growth factor (NGF), brain derived neurotrophic factors (BDNF) neurotrophins 3, 4, 5, (NT-3, NT-4, NT-5)]; and others [colony stimulating factor (CSF), T-cell growth factor, tumor angiogenesis factor (TAF), DNA synthesis promoting factor (DSF), tumor-derived growth factors, fibroblast-derived growth factor (FDGF)].

Receptors that affect growth (that is, receptors for growth-associated ligands) are proteins found associated with cell surfaces that specifically bind their growth factors as ligands. Growth factor receptors are utilized in various clinical and diagnostic applications.

U.S. Patent 4,857,637, issued August 15, 1989, inventors Hammonds et al., describes a method for immunizing an animal against its growth hormone receptor through use of vaccinating with antibodies in order to stimulate growth of the animals.

U.S. Patent 4,933,294, issued June 12, 1990, inventors Waterfield et al., describes studies of structural alterations of the human EGF receptor and its gene and a relationship in tumorigenesis for assays and therapies involving the human EGF receptor. For example, such assays can involve detection of structurally altered or abnormally expressed growth factor receptor and the mRNA transcripts and genes which encode them. EGF may have a role in cell proliferation and differentiation since it induces early eyelid opening and incisor development in new born mice.

U.S. Patent 5,030,576, issued July 9, 1991, inventors Dull et al., describes the role of receptors, such as receptors for growth factors, in designing drugs by the pharmaceutical industry, and discloses use of a

receptor hybrid for screening drug purposes, such as in studies of EGF binding domains.

U.S. Patent 5,087,616, issued February 11, 1992, inventors Myers and Bichon, describes a method for  
5 destroying tumor cells with a composition including a drug conjugate. The conjugate has a growth factor as one moiety and a polymeric carrier with a cytotoxic compound as another moiety. Thus, compositions of the patent are described as binding preferentially to tumor  
10 cells bearing EGF-binding receptors (when an EGF growth factor, for example, is used as a first moiety).

U.S. Patent 5,098,833, issued March 24, 1992, inventors Lasky et al., describes a DNA isolant capable of hybridizing to the epidermal growth factor domain.  
15 Expression systems for recombinant production are said to be useful in therapeutic or diagnostic compositions.

A good background review of a neurotrophic factor related to NGF is provided by WO92/05254, published April 2, 1992, which also describes state of  
20 the art methods of: preparing amino acid sequence variations, site-directed mutagenesis techniques, ligation of coding DNA into a replicable vector for further cloning or for expression, choice of promoters for expression vectors, suitable host cells for expres-  
25 sion, particularly mammalian cells, protein purification upon recovery from culture medium as a secreted protein, derivatization with bifunctional agents to cross-link protein to a support matrix for use with antibodies, entrapment in systems for drug delivery, preparation of  
30 therapeutic formulations, and methods of administration. In addition, preparation of polyclonal and monoclonal antibodies are described, such as are useful in diagnostic assays. These various aspects of isolation, preparation, and applications for a novel neurotrophic  
35 factor, as illustrated by the WO92/05254 publication, are incorporated herein by reference.

Thus, growth factors, their receptors, and DNA or RNA coding sequences therefore and fragments thereof are useful in a number of therapeutic, clinical, research, diagnostic, and drug design applications.

5    Summary of the Invention

In one aspect of the present invention a peptide that can be in substantially purified form is characterized by one or more of the following, highly conserved amino acid sequences:

- 10           QMWLWSQTFCPVLY (SEQ ID NO:3);  
            RFWPRYVKVGSC (SEQ ID NO:4);  
            SKRSCSVPEGMVCK (SEQ ID NO:5);  
            LRWRCQRR (SEQ ID NO:6); and,  
            ISECKCSC (SEQ ID NO:7).

- 15    Peptides of the invention induce dorsal growth in vertebrates and can be prepared in soluble, physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

- 20           In another aspect of the present invention an oligonucleotide, such as cDNA, is provided having substantial similarity to (or being the same as) SEQ ID NO:1 or SEQ ID NO:2. This oligonucleotide can be single or double stranded, be formed of DNA or RNA bases, and can be in the antisense direction with respect to SEQ ID  
25    NOS:1 and 2. SEQ ID NO:1 and SEQ ID NO:2 each code for a functional polypeptide that we have designated "noggin," which is capable of inducing dorsal development in vertebrates when expressed.

- 30           Noggin or fragments thereof (which also may be synthesized by *in vitro* methods) may be fused (by recombinant expression or *in vitro* covalent methods) to an immunogenic polypeptide and this, in turn, may be used to immunize an animal in order to raise antibodies against a noggin epitope. Anti-noggin is recoverable  
35    from the serum of immunized animals. Alternatively,

monoclonal antibodies may be prepared from cells to the immunized animal in conventional fashion. Antibodies identified by routine screening will bind to noggin but will not substantially cross-react with "wnt" or other growth factors. Immobilized anti-noggin antibodies are useful particularly in the diagnosis (*in vitro* or *in vivo*) or purification of noggin.

Substitutional, deletional, or insertional mutants of noggin may be prepared by *in vitro* or recombinant methods and screened for immuno-cross-reactivity with noggin and for noggin antagonist or agonist activity.

Noggin also may be derivatized *in vitro* in order to prepare immobilized noggin and labelled noggin, particularly for purposes of diagnosis of noggin or its antibodies, or for affinity purification of noggin antibodies.

#### Description of the Preferred Embodiments

We have discovered a structurally unique growth factor that is readily available in substantially pure, soluble form. We have named the inventive polypeptide "noggin." This newly isolated neurotrophic factor induces dorsal development in vertebrates.

An earlier described family of proteins that also induces dorsal development are the "wnt" proteins. These, however, in contrast to noggin remain tenaciously bound to cell surfaces. Our initial work with noggin has been in *Xenopus* embryos; however, noggin is highly conserved among vertebrates, as our work with mouse noggin has demonstrated. The prior known FGF growth factor family is also known to be involved in early embryonic induction, but both the FGF proteins and their receptors are distinctly different from noggin. Noggin modifies the actions of FGF (and also activin), for example by potentiating growth, and is thus particularly

suggested in therapeutic compositions for use in combination with other growth factors (as therapeutic adjuvants), such as to modify or potentiate their effects.

5           We have cloned cDNA for noggin. The noggin cDNA contains a single reading frame encoding a 26 kDa protein with a hydrophobic amino-terminal sequence. Noggin is secreted. Noggin's cDNA encodes the protein as a 26 kDa protein, but we have determined that noggin  
10 is secreted in vivo, apparently as a dimeric glycoprotein with a starting apparent molecular weight of about 33 kDa (as the wild-type subunit). When not glycosylated, the monomeric unit has an apparent molecular weight on SDS PAGE of about 25-30 kDa.

15           We have been able to express biologically active noggin in three different systems. Two expression systems we have successfully used to express biologically active noggin have been mammalian cell lines (COS and mouse 293). A third expression system is  
20 injection of synthetic mRNA into *Xenopus* oocytes.

          Expression in these several different systems also illustrates the high degree of conservation for noggin. We have found, for example, substantial sequence similarity between frog noggin and mouse noggin  
25 with a number of completely conserved stretches. Thus, the following amino acid sequences represent completely conserved portions as between frog noggin and mouse noggin:

                  QMWLWSQTFCPVLY (SEQ ID NO:3);  
30                   RFWPRYVKVGSC (SEQ ID NO:4);  
                  SKRSCSVPEGMVCK (SEQ ID NO:5);  
                  LRWRCQRR (SEQ ID NO:6); and,  
                  ISECKCSC (SEQ ID NO:7).

          There is about 87% overall conservation between the  
35 mouse and frog sequences, and we have also observed a unique cysteine distribution between the two. We expect

human noggin will have substantial similarity to mouse noggin and frog noggin.

Noggin nucleic acids, or oligonucleotides, encode a noggin polypeptide or hybridize to such DNA and remain stably bound to it under stringent conditions and are greater than about 10 bases in length; provided, however, that such hybridizing nucleic acid is novel and unobvious over any prior art nucleic acid including that which encodes or is complementary to nucleic acid encoding other growth factors.

By "stringent conditions" we mean are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSo<sub>4</sub> at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

By "substantial similarity," when we are referring to a nucleotide sequence, is meant cross hybridization of sequences under conditions of moderate stringency using a probe greater than 100 nucleotides long at 30°C in a standard buffer (Wahl et al., PNAS, 76, 3683) and washes at 37°C in 300 mM NaCl, 30 mM sodium citrate, 0.2% SDS at pH 7. Alternatively, one is able to isolate, by polymerase chain reaction, a fragment of DNA coding for noggin or noggin family members when using primers of degenerate sequence that encode those SEQ ID NOS:3-7.

By "substantial similarity" when reference is made to proteins is that noggin from different species, or noggin family members within a species, will preserve the positions of cysteine residues in at least 80% of positions throughout the protein. Like the neurotrophin family, the sequence of the mature form of noggin and

noggin related polypeptides will be identical in at least 40% of positions. Substantial similarity at the protein level includes an ability of a subject protein to compete with noggin for binding to receptors and some  
 5 (but not all) monoclonal antibodies raised against noggin epitopes.

The cloned cDNA for noggin (derived from frog) is designated herein as SEQ ID NO:1 and from mouse as SEQ ID NO:2. We have used RNA transcripts from the SEQ  
 10 ID NO:1 clone to rescue embryos and return them to substantially normal development when the noggin RNA is injected into ventralized embryos. In high doses this results in excessive head development and it is for this reason we named the protein "noggin." In northern blot  
 15 analysis the noggin cDNA hybridizes to two mRNAs that are expressed both maternally and zygotically.

When using nucleotide sequences coding for part or all of noggin in accordance with this invention, the length of the sequence should be at least sufficient  
 20 in size to be capable of hybridizing with endogenous mRNA for the vertebrate's own noggin. Typically, sufficient sequence size (for example, for use as diagnostic probes) will be about 15 consecutive bases (DNA or RNA). In some diagnostic and therapeutic  
 25 applications, one may wish to use nucleotide noggin coding sequences (analogous to all or a portion of SEQ ID NO:1 or SEQ ID NO:2) in the anti-sense direction with respect to either SEQ ID NOS:1 or 2.

We suggest as a few preferred primers for  
 30 amplifying noggin from other species (e.g. human):

5' Primer 1

C A A/G A C N T T C/T T G C/T C C N G T N

5' Primer 2

T T C/T T G G C C N C/A G N T A C/T G T N A A A/G  
 35 G T N G G

5' Primer 3

C C N G A A/G G G N A T G G T N T G

3' Primer 1

C A N C/G T/A A/G C A C/T T T A/G C A C/T T C

5

3' Primer 2

C A N A C C A T N C C C/T T C N G G

3' Primer 3

C G/T N C G/T T/C T G G/A C A N C G/T C C A

where N represents a mixture of all four nucleotides and  
 10 mixtures of two nucleotides are represented by  
 alternates (e.g. A/G).

Although noggin transcript is not localized in  
 the oocyte and cleavage stage embryo, zygotic  
 transcripts are initially restricted to the presumptive  
 15 dorsal mesoderm, and reach their highest levels at the  
 gastrula stage in the dorsal lip of the blastopore  
 (Spemann's organizer). In the neurula, noggin is  
 transcribed in the notochord and prechordal mesoderm.

Without being bound by theory, we have  
 20 formulated hypotheses about the embryological effects of  
 noggin based on where it is expressed, and on the  
 effects of RNA injection in embryos. Since noggin is  
 expressed in the spemann organizer, we believe noggin to  
 be a mediator of the effects of the Spemann organizer,  
 25 namely neural induction and dorsalization of the  
 mesoderm. Since noggin is expressed in the notochord  
 and head mesoderm, we believe noggin to influence either  
 the dorsal-ventral pattern or anterior-posterior pattern  
 of the neural plate. Since noggin is expressed in the  
 30 branchial arch neural crest, we believe it may therefore  
 influence whether neural crest cells deposit cartilage  
 and also to influence later branchial arch growth and  
 remodelling. Noggin is expressed in the tail fin neural  
 crest, and since neural crest is required for growth of  
 35 the fin, noggin may act as a growth factor for epidermis  
 or mesenchyme.

Although much of our experimental work has involved rescue of embryonic development, because expression in the notochord persists in the growing tail bud and a discontinuous line of stained cells (indicating expression of noggin initiated at new sites) runs the length of the roof plate of the neural tube (and is also apparent in the head mesoderm), we believe noggin is expressed as an adult cell function also.

A number of applications for noggin are suggested from its properties.

The noggin cDNA should be useful as a diagnostic tool (such as through use of antibodies in assays for proteins in cell lines or use of oligonucleotides as primers in a PCR test to amplify those with sequence similarities to the oligonucleotide primer, and to see how much noggin is present, e.g. primers such as 5' Primers 1-3 and 3' Primers 1-3).

Because noggin has a pattern of expression that suggests it is used to regulate cartilage production in the embryonic head, clinical uses to regulate cartilage and bone growth are suggested for noggin in therapeutic compositions and particularly in combination with other growth factors due to a property of noggin to potentiate at least some growth factors.

Since neural crest cells are required for the tadpole fin to grow, noggin seems to be a growth factor for the tissue matrix and epidermis and should prove useful, for example, in wound healing compositions.

Noggin, of course, provides the key to isolate its receptor. Since many receptors mutate to cellular oncogenes, the noggin receptor should prove useful as a diagnostic probe for certain tumor types. Thus, when one views noggin as ligand in complexes, then complexes in accordance with the invention include antibody bound to noggin, antibody bound to peptides derived from noggin, noggin bound to its receptor, or peptides

derived from noggin bound to its receptor. Mutant forms of noggin, which are either more potent agonists or antagonists, are believed to be clinically useful. Such complexes of noggin and its binding protein partners  
5 will find uses in a number of applications.

Practice of this invention includes use of an oligonucleotide construct comprising a sequence coding for noggin and for a promoter sequence operatively linked to noggin in a mammalian or a viral expression  
10 vector. Expression and cloning vectors contain a nucleotide sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromo-  
15 somes, and includes origins of replication or autonomously replicating sequences. The well-known plasmid pBR322 is suitable for most gram negative bacteria, the 2 $\mu$  plasmid origin for yeast and various viral origins (SV40, polyoma, adenovirus, VSV or BPV)  
20 are useful for cloning vectors in mammalian cells.

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. Typically, this is a gene that encodes a protein necessary for the survival or growth of a host cell  
25 transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or  
30 other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies.

Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR) or thymidine kinase. Such markers enable the identifica-  
35 tion of cells which were competent to take up the noggin nuclei acid. The mammalian cell transformants are

placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of noggin can therefor be synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium which contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Nat. Acad. Sci.*, 77, 4216 (1980). The transformed cells then are exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding noggin. Alternatively, host cells transformed by an expression vector comprising DNA sequences encoding noggin and aminoglycoside 3' phosphotransferase (APH) protein can be selected by cell growth in medium containing an aminoglycosidic antibiotic such as kanamycin or neomycin or G418. Because eukarotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as neo resistant genes, may be used as dominant selectable markers in a wide range of eukaryotic host cells, by which cells transformed by the vector can readily be identified.

Expression vectors, unlike cloning vectors, should contain a promoter which is recognized by the host organism and is operably linked to the noggin nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters can be operably linked to noggin encoding DNA by removing them from their gene of origin by restriction enzyme digestion, followed by insertion 5' to the start codon for noggin.

Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

Transcription of noggin-encoding DNA in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma, cytomegalovirus, adenovirus, retroviruses, hepatitis-B virus, and  
5 most preferably Simian Virus 40 (SV40), or from heterologous mammalian promoters, e.g. the actin promoter. Of course, promoters from the host cell or related species also are useful herein.

Noggin is believed to find use as an agent for  
10 enhancing the survival or inducing the growth of nerve and muscle cells. It, therefore, is useful in the therapy of degenerative disorders of the nervous system ("neurodegenerative diseases"), including such diseases as Alzheimer's disease, Parkinson's disease,  
15 Huntington's chorea, ALS, peripheral neuropathies, and other conditions characterized by necrosis or loss of neurons, whether central, peripheral, or motoneurons. In addition, it may be useful for treating damaged nerve cells, e.g., nerves damaged by traumatic conditions such  
20 as burns and wounds, diabetes, kidney dysfunction, and the toxic effects of chemotherapeutics used to treat cancer and AIDS. It also is useful as a component of culture media for use in culturing nerve cells *in vitro*.

Practice of this invention includes  
25 preparation and uses of a diagnostic or therapeutic agent comprising a nucleotide sequence of at least about 15 DNA or RNA bases analogous to all or a portion of either SEQ ID NO:1 or SEQ ID NO:2. That is, noggin preparations are useful as standards in assays for  
30 noggin and in competitive-type receptor binding assays when labelled with radioiodine, enzymes, fluorophores, spin labels, and the like. Therapeutic formulations of noggin are prepared for storage by mixing noggin having the desired degree of purity with optional physiologically acceptable carriers, excipients or  
35 stabilizers, in the form of lyophilized cake or aqueous

solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; anti-oxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or PEG.

Inventive complexes comprise a ligand characterized by one or more of the SEQ ID NOS:3-7. The ligand can be bound to a protein, such as antibody. Such antibodies can be polyclonal or monoclonal.

Polyclonal antibodies to noggin generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of noggin and an adjuvant. It may be useful to conjugate noggin or a fragment containing the target amino acid sequence to a protein which is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxy-succinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ .

Animals can be immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally in multiple sites. One month

later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is  
5 assayed for anti-noggin titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same noggin polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates  
10 also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and  
15 immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by EB virus transformation and screening for clones expressing the desired antibody.

Noggin antibodies are useful in diagnostic  
20 assays for noggin or its antibodies. In one embodiment of a receptor binding assay, an antibody composition which binds to all of a selected plurality of members of the noggin family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized  
25 antibody composition in order to adsorb all noggin family members, and then the immobilized family members are contacted with a plurality of antibodies specific for each member, each of the antibodies being individually identifiable as specific for a predetermined  
30 family member, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member can be determined.

35 Noggin antibodies also are useful for the affinity purification of noggin from recombinant cell

culture or natural sources. Noggin antibodies that do not detectably cross-react with other growth factors can be used to purify noggin free from these other family members.

5                   Aspects of the invention will now be illustrated by the following examples.

### EXPERIMENTAL PROCEDURES

#### Production of *Xenopus* embryos

10                   *Xenopus* embryos were prepared by the protocol described by Condie and Harland (*Development*, 101, 93-105, 1987). Embryos were staged according to the table of Nieuwkoop and Faber ("Normal Table of *Xenopus laevis*" (Daubin), Amsterdam: North Holland, 1967). Ventralized embryos were produced by UV irradiation with a  
15                   Statalinker (Stratagene), and dorsalized embryos were produced by treatment with LiCl as described by us in our paper on certain "wnt" proteins (designated "Xwnt-8"), Smith and Harland, *Cell*, Vol. 67, pp. 753-765 (1991) (incorporated by reference and occasionally  
20                   referred to hereinafter as "S&H, supra").

### EXAMPLE 1

#### Isolation and Sequencing of Noggin cDNA

25                   The construction of the size-selected plasmid cDNA library from stage 11 LiCl-treated embryos was as follows. Sixty micrograms of poly(A)<sup>+</sup> RNA from stage 11 LiCl-treated embryos was size fractionated on a 10% to 30% sucrose gradient in the presence of methylmercuric hydroxide. First strand cDNA was synthesized from 2 µg of the size-fractionated poly(A)<sup>+</sup> RNAs primed with  
30                   oligo(dT) oligonucleotide containing the recognition site for NotI. After synthesis of the second strand, cDNAs were treated with EcoRI methylase, ligated with

EcoRI linkers, digested with EcoRI and NotI, and finally ligated to 125 ng of modified pGEM-5Zf(-) (Promega). The pGEM-5Zf(-) used here was modified by the addition of an oligonucleotide into the NsiI site to create an EcoRI site. The vector was not treated with alkaline phosphatase, but the excised polylinker sequence was removed on a sepharose 4BCL column. The ligated products were used to transform XL-I Blue cells (Stratagene), and plated to give 100,000 colonies per 15 cm plate. Plasmid DNAs were isolated from plate cultures by the alkaline-lysis/polyethylene glycol precipitation protocol.

Dorsalizing activity in the library was assayed by injecting RNA transcripts made from pooled plasmid DNA. Single clones were isolated by a process of sib selection. In this procedure the plasmid library was replated on 12 plates with 10-fold fewer colonies per plate. RNA was synthesized from pooled plasmid DNAs isolated from each plate and tested for dorsalizing activity by injection into UV-ventralized embryos. Those pools with dorsalizing activity were replated and screened as described above. This process was repeated until single clones were isolated.

*In vitro* RNA synthesis, injection assay for dorsal axis rescue and sib-selections were also done, as described by us in S&H, *supra*.

The nucleotide sequence of both strands of the isolated noggin cDNA clone was determined by the dideoxy termination method using modified T7 DNA polymerase (US Biochem). Deletions were prepared in sequencing templates by both restriction enzyme and exonuclease III digestion (Henikoff, *Meth. Enzymol*, 155, 156-165, 1987).

#### *In vitro* translation

One-half  $\mu$ g of *in vitro* synthesized noggin, Xwnt-8, and goosecoid mRNAs were translated in a

nuclease treated rabbit reticulocyte lysate (Promega) with added <sup>35</sup>S-methionine according to the manufacturer's instructions. The translation products were visualized by SDS-polyacrylamide gel electrophoresis (12% gels) followed by fluorography. Noggin protein had the molecular weight predicted by the open reading frame.

#### RNA Isolation and Analysis

Total RNA was isolated from embryos and oocytes by a small scale protocol as described by Condie and Harland, *supra*. Dorsal lips were dissected from 30 unfixed stage 10.5 embryos and pooled for total RNA preparation. Samples containing either the total RNA equivalent of 2.5 embryos or approximately 2 µg of poly A+ RNA were analyzed by northern blotting. Random primed DNA probes were prepared from a 1,323 bp fragment of noggin cDNA from the EcoRI site at nucleotide -83 to an EcoRV sites that lies in the vector immediately 3' to the end of the cDNA.

RNAse protection assays were done using a protocol as detailed by Melton et al. (*Nuc. Acids Res.*, 12, 7035-7056, 1984) with minor modifications (C. Kintner, Salk Institute, La Jolla, California). A noggin cDNA exonuclease III deletion clone, illustrated by SEQ ID NO:1 but having a deletion from the 3' end to nucleotide 383, was used as a template for synthesizing RNA probes. The template DNA was linearized by Eco RI restriction enzyme digestion and a 463 base antisense RNA incorporating <sup>32</sup>P was synthesized with T7 RNA polymerase. A 387 base antisense EF1α RNA probe was used as a control for amount of RNA per sample. Probes were gel purified prior to use.

#### In situ hybridization

After fixation and storage, the embryos were checked to ensure the blastocoel and archenteron were

punctured. Care was taken to puncture the residual blastocoel of neurulae and tadpoles as well as the archenteron. Embryos were rewashed at room temperature in 100% ethanol for two hours to remove residual lipid.

5 After hybridization, staining was allowed to develop overnight and the embryos were then fixed in Bouin's. Newly stained embryos have a high background of pink stain but most of this washes out, leaving the specific stain. Following overnight fixation, the embryos were

10 washed well with 70% ethanol, 70% ethanol buffered with PBS and methanol. Embryos were cleared in Murray's mix and photographed with Kodak Ektar 25 film, using a Zeiss axioplan microscope (2.5 or 5x objective with 3x12B telescope to assist with focusing).

15 Lineage tracing

Lineage tracing with mRNA that encodes nuclear localized  $\beta$ -galactosidase was as we described in S&H, *supra*. Ventralized embryos were coinjected at the 32 cell stage with 0.5 ng  $\beta$ -galactosidase and 25 pg noggin

20  $\Delta 5'$  mRNAs. Embryos were fixed and stained with X-gal at approximately stage 22.

Noggin cDNA Encodes a Novel Polypeptide

The 1833 nucleotide sequence of the selected clone is shown by SEQ ID NO:1 and sometimes also

25 referred to as "clone A3." The sequence contains a single long open reading frame encoding a 222 amino acid polypeptide with a predicted molecular weight of 26 kDa. At the amino terminus, the hydrophobic stretch of amino acids suggests that the polypeptide enters the secretory

30 pathway. There is a single potential site for N-linked glycosylation at amino acid 61. Extensive untranslated regions are located both 5' and 3' to the reading frame (593 and 573 bp, respectively). The 3' untranslated region is particularly rich in repeated dA and dT

nucleotides, and contains, in addition to a polyadenylation signal sequence located 24 bp upstream from the start of the poly A tail, a second potential polyadenylation sequence 147 bp further upstream.

5               Sense RNA synthesized from clone A3 with SP6 RNA polymerase was translated in a rabbit reticulocyte lysate system. The <sup>35</sup>S-labeled products were fractionated on a 12% SDS-polyacrylamide gel and visualized by fluorography. The major protein product had the  
10               expected molecular weight of approximately 26 kDa.

              Comparison of the amino acid sequence of the predicted polypeptide to the National center for Biotechnology Information BLAST network (non-redundant data base) did not identify any similar sequence. Thus,  
15               this clone encodes the new type of protein we have named "noggin," which is secreted, and which has dorsal inducing activity in *Xenopus*.

#### Noggin mRNA can Rescue a Complete Dorsal-Ventral Axis

              Injection of noggin RNA into a single  
20               blastomere of a four cell stage UV-ventralized embryo can restore the complete spectrum of dorsal structures. The degree of axis rescue was dependent upon the amount of RNA injected, with embryos receiving low doses having only posterior dorsal structures, while embryos  
25               receiving higher doses had excess dorsal-anterior tissue. RNA transcripts from two noggin plasmids were tested. The first contained the full cDNA. The second (pNogginΔ5') had a deletion removing the first 513 nucleotides of the 5' untranslated region up to the Eco  
30               RI site. The resulting embryos from injection of RNA transcripts of these two plasmids, as well as Xwnt-8 RNA for comparison, were scored according to the dorso-anterior index (DAI) scale of Kao and Elinson (*Dev. Biol.*, 127, 64-77, 1988). In this scale, a completely  
35               ventralized embryo is scored as zero, a normal embryo is

scored as 5, and the most severely dorsoanteriorized embryos, those having radial dorsoanterior structures, were scored as 10. RNA synthesized from pNoggin $\Delta$ 5' (noggin $\Delta$ 5' mRNA) repeatedly gave a higher DAI than the  
5 equivalent amount of mRNA synthesized from the complete cDNA. The dose-dependency of axis rescue by noggin $\Delta$ 5' mRNA was very similar to that of Xwnt-8 mRNA.

UV treated embryos were also injected with a higher doses (1,000 pg) of the noggin mRNAs. Injection  
10 of this dose of noggin mRNA into one blastomere at the four cell stage resulted in embryos with very severe hyperdorsalization (DAI >7). However, most of these embryos died at the late gastrula/early neurula stage. Apparently excessively strong gastrulation movements  
15 resulted in the thinning and rupture of the blastocoel roof. We have also observed this effect with high doses of injected Xwnt-8 mRNA.

The rescue of dorsal development by both noggin $\Delta$ 5' and Xwnt-8 mRNAs followed a consistent pattern  
20 in which increasing amounts of the mRNAs lead to progressively more anterior structures being rescued. For example, embryos that received 1 pg of the RNAs had primarily the posterior and trunk dorsal structures rescued, and for the most part lacked head structures.  
25 Higher doses (10 or 100 pg) of both of the RNAs resulted in embryos with more anterior development, and many had either nearly normal or hyperdorsalized phenotypes.

#### Noggin Injected Blastomeres Act as a Nieuwkoop Center

The effect of varying the site of noggin mRNA  
30 injection was investigated. Thirty-two cell stage UV-treated embryos were injected with either 0.5 ng of  $\beta$ -galactosidase mRNA alone or 0.5 ng  $\beta$ -galactosidase mixed with 25 pg noggin $\Delta$ 5' mRNA. Injection of noggin mRNA into blastomeres of the vegetal tier gave the most  
35 strongly dorsoanteriorized embryos. In both of the

vegetal injected embryos the nuclear X-Gal staining was found almost exclusively in the endoderm (the mRNA encodes a  $\beta$ -galactosidase that translocates to the nucleus, allowing distinction from the diffuse background stain). One of the embryos shown was strongly hyperdorsalized (DAI approximately 7) as a result of the noggin mRNA injection, and had a severely truncated tail and enlarged head structures. Embryos were also rescued by noggin mRNA injections into the marginal zone. In these embryos  $\beta$ -galactosidase staining was observed primarily in the axial and head mesoderm. Injection of noggin mRNA into the animal pole had very little effect on axis formation. Likewise,  $\beta$ -galactosidase mRNA alone was without effect.

15 Noggin mRNA is Expressed Both Maternally and Zygotically

In northern blot analysis of RNA from *Xenopus* embryos two noggin mRNA species of approximate sizes 1.8 and 1.4 kb were observed. A relatively low level of noggin mRNA was detected in oocytes. By stage 11 the level of noggin mRNA was significantly higher, reflecting zygotic transcription (as opposed to the maternally deposited transcripts seen in oocytes). Noggin mRNA remained at the elevated level up to the latest stage examined (stage 45).

25 We expect that the primary dorsalizing RNA in our library to be elevated in LiCl-treated embryos relative to normal or UV-treated embryos. Lithium ion treatment resulted in a large increase in the amount of noggin mRNA expressed, relative to untreated embryos. 30 UV treatment had the opposite effect. Noggin mRNA expression was essentially undetectable in total RNA samples from these embryos. Thus, the abundance of noggin mRNA in manipulated embryos parallels the rescuing activity.

We analyzed the distribution of noggin mRNA in oocytes and cleavage stage embryos. Since the amount of maternally deposited noggin RNA is too low for *in situ* hybridization to detect above background, we used an  
5 RNase protection assay. Oocytes were dissected into animal and vegetal halves. No enrichment of noggin mRNA was seen in either hemisphere relative to total oocyte RNA. Four-cell stage embryos were dissected into dorsal and ventral halves, as well as animal and vegetal  
10 halves. Noggin transcripts were found to be distributed evenly between dorsal and ventral hemispheres as well as animal and vegetal hemispheres. The same result was obtained with embryos that were tilted 90° immediately following fertilization and then marked with a vital dye  
15 on their uppermost side to indicate the future dorsal side. Older (32 cell stage) blastula embryos were also dissected into dorsal-ventral and animal-vegetal halves. No enrichment of noggin mRNA in any of the hemispheres was seen relative to the total embryo. In addition, UV  
20 treatment did not alter the abundance of maternally deposited noggin RNA, indicating no preferential degradation in ventral tissues. Samples with known amounts of *in vitro* synthesized noggin mRNA were included in the RNase protection assay. From these and  
25 other data we estimate that there is approximately 0.1 pg of noggin mRNA per blastula stage embryo and 1 pg per gastrula stage embryo.

The localization of noggin transcripts was investigated in early gastrula stage embryos. Dorsal  
30 lips were dissected from stage 10.5 embryos. A northern blot of equal amounts of total RNA from intact embryos, dissected dorsal lips, and from the remaining embryo after dissection of the dorsal lip was hybridized with a noggin probe and then re-hybridized with an EF1 $\alpha$   
35 probe, as a control for amount of RNA loaded per sample.

The autoradiograph of the blot showed that noggin mRNA at this stage is enriched in the dorsal lip.

In situ Hybridization; Zygotic Expression of Noggin in the Spemann Organizer

5                   The localization of noggin transcripts in developing embryos was examined in greater detail using whole mount *in situ* hybridization. Whole fixed embryos were hybridized with digoxigenin containing RNA probes. Hybridized RNA probe was then visualized with an  
10 alkaline phosphatase-conjugated anti-digoxigenin antibody. The specificity of hybridization seen with antisense noggin probes was tested both by hybridizing embryos with sense noggin probes, and by using two non-overlapping antisense probes. Due both to the low level  
15 of expression, and to background staining, noggin mRNA could not be detected unequivocally before the late blastula stage. The increased level of noggin mRNA that was detected by northern blot following activation of zygotic transcription was apparent in *in situ* hybridi-  
20 zation at stage 9 as a patch of staining cells on the dorsal side of the embryo. Viewed from the vegetal pole, this patch of cells was restricted to a sector of about 60°. A side view of the same embryo shows that the staining cells were located within the marginal zone  
25 (i.e., between the animal and vegetal poles and within the presumptive dorsal mesoderm forming region). Transcripts are largely restricted to the nucleus at this stage.

                  A side view of an early gastrula stage embryo  
30 (approximate stage 10.5 shows specific hybridization primarily in the involuting mesoderm at the dorsal lip. A vegetal view of the same embryo (blastopore lip arrowed) shows that noggin mRNA is most abundant on the dorsal side, but expression extends at the lower level  
35 to the ventral side of the embryo. This method if in

situ hybridization does not detect transcripts in the most yolky endodermal region of embryos, therefore we cannot rule out expression in more vegetal regions than those seen in the Figure. Treatments which are known to affect the size of the dorsal lip (LiCl treatment, UV irradiation) had a profound effect on the pattern of *noggin* in situ hybridization. In LiCl treated embryos the staining is intense throughout the marginal zone. UV treatment reduced the hybridization signal to low levels. This result is consistent with amounts of *noggin* mRNA seen by northern blot analysis. The UV treated embryo also is a negative control for specificity of hybridization.

As gastrulation proceeds, *noggin* mRNA staining follows the involuting dorsal mesoderm, and is highest in the presumptive notochord. By the late neurula stage (approximately 18) *noggin* mRNA expressing cells are clearest in the most dorsal mesoderm, primarily in the notochord but also extending more anteriorly into the pre-chordal mesoderm. The anterior tip of the notochord is arrowed. During tailbud stages expression of *noggin* in the dorsal mesoderm declines, though expression in the notochord persists in the growing tailbud. Expression of *noggin* initiates at several new sites, which become progressively clearer as the tadpole matures. A discontinuous line of stained cells runs the length of the roof plate of the neural tube. Staining is also apparent in the head mesoderm, primarily in the mandibular and gill arches. We suspect that this expression corresponds to skeletogenic neural crest cells. Furthermore, subsets of these cells express homeobox genes that mark different anterior-posterior levels of the head neural crest, for example *En-2* in the mandibular arch is seen by antibody staining. Cells with stellate morphology stained from *noggin* mRNA in the tail fin. These stellate cells are also likely to be

derived from the neural crest. None of these patterns were seen with the sense probe, or with a number of other probes.

## EXAMPLE 2

### 5 Noggin cDNA Transfected into COS Cells Produces Active Conditioned Medium

For COS cells the noggin cDNA was inserted into a COS cell expression vector. COS cells were transfected, and medium harvested after allowing  
10 expression of the introduced noggin genes. This medium has been tested in an animal cap assay for mesoderm-inducing or dorsalizing activity. We have tested two transfection protocols, a standard one, where cells recover and then are transferred to serum-free medium,  
15 and an alternate where cells are transferred to a defined medium lacking serum but containing transferrin, insulin, and BSA. Cells remain healthy in the supplemented medium and a cotransfected  $\beta$ -galactosidase gene gives 100 fold more activity than in the unsupplemented  
20 medium. The results of treating cells with these media is shown below in Table 1. Animal caps were taken from ventralized animals, treated and at the end of neurulation they were scored for elongation, usually a sign that notochord or neural tissues have been induced.  
25 Elongation is indicated in Table 1 by a "+" and even greater elongation a "++." In addition, they are scored for a molecular marker by Northern blotting.

As shown by the data of Table 1, the noggin cDNA has a large effect on the COS cell conditioned  
30 medium. However, noggin is probably interacting with something else in the medium, since COS-cell conditioned medium alone has some activity. It is possible that noggin is causing the cells to secrete something that they normally would not, but the experiments do indicate

that noggin is secreted and is responsible for some of the activity.

TABLE 1

COS Cell Conditioned Medium: Effects on Animal Caps

5		Elongation	N-CAM expression
	Transferred to serum free medium + transferrin, BSA, and insulin		
10	1. Vector only	+/-	+
	2. Noggin cDNA	++	++
	Transferred to serum free medium without supplements		
15	1. Vector only	-	-
	2. Noggin cDNA	-	-

Noggin mRNA Injected into Oocytes Produces Active Secreted Noggin Protein

20                   A second approach to studying whether the protein can be secreted in active form is to inject oocytes with mRNA and take material secreted by the oocyte. A particular advantage of this method is that the injected mRNA is efficiently translated, and most of

25 the translation of the oocyte can be taken up by the injected mRNA. A new protein, whose synthesis is directed by injected noggin mRNA is secreted into the medium. Noggin clearly synergizes with activin to produce elongated explants that express elevated levels

30 of muscle actin.

Biochemical Properties of Noggin

                  Injected oocytes are injected with mRNA, and labelled with <sup>35</sup>S methionine. Most of the radioactive

protein secreted into the medium is from the injected mRNA. The noggin protein, which is almost isotopically pure, can then be analyzed. From this analysis we have determined that noggin is a dimeric glycoprotein. When  
5 run under reducing conditions, and treated with N-glycanase to remove sugar residues, noggin migrates only slightly slower than its predicted molecular weight of 26 kDa. The removal of sugar side chains results in a loss of about 4 kDa from a starting apparent molecular  
10 weight of 33 kDa. When run under non-reducing conditions it migrates at double this value.

We do not yet know if the dimer of 30 kDa protein is the active species, or if there is a proteolytically processed form which is active. In a  
15 control experiment with activin mRNA, oocytes produce activin activity, but the bulk of the radiolabelled protein migrates as the precursor form. Only a small amount of processed protein (15 kDa) was detected. It is possible that noggin injected oocytes secrete  
20 predominantly unprocessed protein and a trace of extremely active processed protein that we have not detected. Despite the caveats, the main point from analysis of injected oocytes and transfected COS cells is that active noggin can be obtained as a freely  
25 soluble secreted polypeptide. This sets it apart from the other group of genes with dorsalizing activity, the wnts. Wnt proteins have not been available in soluble form and this has greatly hampered the analysis of their biological activities, and of the receptor that binds to  
30 them.

### EXAMPLE 3

#### Cloning of the Mouse Noggin Homolog

It is currently impossible to eliminate zygotic noggin transcription from developing *Xenopus*

embryos. In contrast, it should be possible to generate homozygous null mutations in the mouse. We have cloned the mouse noggin cDNA (SEQ ID NO:2). This is useful to generate mutant mice. In addition to generating the probes and tools to make mutant mice, a comparison of the noggin sequences should be a useful predictor of conserved domains and functions. The C-terminal 80 amino acids are 87% identical between SEQ ID NOS:1 and 2.

10                    Mouse noggin was isolated from an embryonic cDNA library by probing with a radiolabelled frog noggin cDNA under conditions of moderate stringency (as defined earlier). Subsequently a genomic clone was isolated by probing a genomic library with the mouse noggin cDNA  
15                    under conditions of high stringency (as defined, but hybridized at 42° C and washed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate).

---

20                    It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Harland, Richard M.  
Smith, William C.
- (ii) TITLE OF INVENTION: DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Majestic, Parsons, Siebert & Hsue
  - (B) STREET: Four Embarcadero Center, Suite 1450
  - (C) CITY: San Francisco
  - (D) STATE: California
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 94111-4121
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 07/939,954
  - (B) FILING DATE: 03-SEP-1992
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Siebert, J. Suzanne
  - (B) REGISTRATION NUMBER: 28,758
  - (C) REFERENCE/DOCKET NUMBER: 2900.3
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (415) 362-5556
  - (B) TELEFAX: (415) 362-5418
  - (C) TELEX: 278638 MGPS

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1834 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Xenopus laevis*

(D) DEVELOPMENTAL STAGE: Gastrula

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Smith, William C.

Harland, Richard M.

(B) TITLE: Expression Cloning of noggin, a New  
Dorsalizing Factor Localized to the Spemann  
Organizer in *Xenopus* Embryos

(C) JOURNAL: Cell

(D) VOLUME: 70

(F) PAGES: 829-840

(G) DATE: Sept. 4-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTAATAAATC CTAAGTAGCC AGAGGGACGA GCTACAGACT GGTGCGGCG CAGGGTTTAT  
60

CCAGGGCAGA GAGGAGCAGC AAAAGCACAT TGCGCAGCTC TCACTCCCCC TTCCTTCTG  
120

CTTCACTCTA TAAGGGCTCC TGCAAATGAA AGAGACCTGC GGGGATTGCG GCGGACAGAT  
180

GTAAAGGAGA TCCTGCAACT TTCTCTGGTT GCATCCCTGG GAGTCGCTGC GCGCCGCTGG  
240

CTGATTGCGA CTGTTGCTTT CCACAGCTCC CTTCTTCCGC AGTTTCTTCT AGGAGCAGAT  
300

CGAGTCTCTG GTTACCATGG TGATCGAGCT GAAAGTGAAG AATATTTAAG AGAGGGGAGG  
360

CTGGAGCCAG CAGGCAGACA AAGTGGTGCC ACCAAGGACT GTGCGTAAAG GGTGAGCGCA  
420

TTGGAGACAG ACAGGGGCTC TGCTGAACTT CCACTTGACT GCGATGAGAG GGGGGAATCC  
480

CCAATTCGCT AGGTGCCCCT GAACCCCCCA GAATTCCTCC TCTGATGCAT TATTTATGAT  
540

CTCTGGCAAG AAATCGGGAG CACCCAACCTC TTATTTTGTG CAGCTGTGTG CAGCATGGAT  
600

CATTCCCAGT GCCTTGTGAC TATATATGCT CTGATGGTCT TCTTGGGACT TAGAATAGAC  
660

**SUBSTITUTE SHEET**

CAAGGGGGTT GCCAACATTA TCTGCACATC AGACCGGCTC CTAGTGAAAA CCTACCACTG  
720

GTGGACCTTA TTGAGCACCC GGATCCCATC TATGATCCCA AGGAGAAGGA TCTTAACGAG  
780

ACCTTGCTGA GGACTTTAAT GGTGGACAC TTGACCCCA ACTTTATGGC CACCATCCTG  
840

CCAGAGGAGA GACTTGGAGT GGAGGACCTT GGGGAGTTGG ATCTCCTTCT TAGGCAGAAG  
900

CCCTCGGGGG CAATGCCAGC GGAAATCAAG GGACTGGAGT TTTACGAGGG GCTTCAGAGC  
960

AAAAAGCACA GACTGAGCAA GAAACTCAGG AGAAAGTTGC AGATGTGGCT CTGGTCCCAG  
1020

ACCTTCTGTC CTGTCCTTTA CACATGGAAT GACCTAGGGA CCAGGTTTTG GCCTCGCTAT  
1080

GTGAAAGTAG GGAGCTGCTA CAGTAAGAGG TCTTGTTCTG TGCCAGAGGG CATGGTTTGC  
1140

AAAGCTGCCA AGTCTATGCA TTGACCATC TTAAGGTGGA GATGTCAACG CAGGGTTCAG  
1200

CAGAAGTGTG CGTGGATAAC CATTGAGTAC CCTGTCATTT CCGAGTGCAA ATGCTCATGC  
1260

TGAGACTCTT GGACTAATGC AAAAAGACAG TAGCTTCATG GTTCAAGCTT CATGTTATAT  
1320

GCACTGTAAT ATGTAGAAAT GTATATGTGT GTATATATGG CATTGGTCTA AATTACTATT  
1380

AAAAGGTCAG TATTATTCTT TTAAATAACC AGTGTCTACT GTATTTCCAA CACTATTATC  
1440

CTGGTTGTGT TTTATTTTAA TAATATTATT ATTATTTTTT TTTTGCCTAA TGTATCTCTA  
1500

TTTATATCCA AAAAAAGAGC ACTTCGCTTG GCGAAGCATT TTTTTTTAAA GAAAAAAAAA  
1560

ACAAATTTAA TAGTTTAATA ATATAGAAGC ATTTTTTTCC TTTAATGGAA AATGTGCCTT  
1620

TTTTTGATGG ACCTCAAAAA AAAAATGAAT AAAAACCAGA GCAAGATATA ATTTTCAGTT  
1680

TATTGTACAT AGAAAGAGAA CACATTTTGA AAGATGAAAA ATTTTACTCT TGTAATGAG  
1740

34

AACTATCTGC TATTTATTCT TTTATTTTTT TTTTCCTCCC CCTGTAGAGT GCAGAATAAA  
1800

AGTAAACCAC TAAAATATTA AAAAAAAAAA AAAA  
1834

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 356 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mus musculus
- (D) DEVELOPMENTAL STAGE: Gastrula

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCAGATGTGG CTGTGGTCAC AGACCTTCTG CCCGGTGCTG TACGCGTGGA ATGACCTAGG  
60

CAGCCGCTTT TGGCCACGCT ACGTGAAGGT GGGCAGCTGC TTCAGCAAGC GCTCCTGCTC  
120

TGTGCCCCGAG GGCATGGTGT GTAAGCCATC CAAGTCTGTC CACCTCACGG TGCTGCGGTG  
180

GCGCTGTCAG CGGCGCGGGG GTCAGCGCTG CGGCTGGATT CCCATCCAGT ACCCCATCAT  
240

TTCCGAGTGT AAGTGTTTCCT GCTAGAACTC GGGGGGGCCC CCTGCCCGCG CCCAGACACT  
300

TGATGGATCC CCACCAACGN CCCCCCTACC CCCACCACCT CCAACCAGTT TCACCA  
356

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

**SUBSTITUTE SHEET**

35

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln	Met	Trp	Leu	Trp	Ser	Gln	Thr	Phe	Cys	Pro	Val	Leu	Tyr
1				5					10				

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg	Phe	Trp	Pro	Arg	Tyr	Val	Lys	Val	Gly	Ser	Cys
1				5					10		

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser	Lys	Arg	Ser	Cys	Ser	Val	Pro	Glu	Gly	Met	Val	Cys	Lys
1				5					10				

36

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 8 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Arg Trp Arg Cys Gln Arg Arg  
1                    5

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 8 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Ser Glu Cys Lys Cys Ser Cys  
1                    5

It Is Claimed:

1. A substantially pure polypeptide characterized by a physiologically active, soluble form, the polypeptide capable of inducing dorsal development in vertebrates.

2. The polypeptide as in claim 1 being from an animal species, and being isolated from a recombinant expression system or from the animal species free of contaminating polypeptides of said animal species.

3. The polypeptide as in claim 1 being glycosolated or unglycosolated.

4. A composition having a peptide characterized by one or more of the following sequences:

QMWLWSQTFCPVLY (SEQ ID NO:3);

RFWPRYVKVGSC (SEQ ID NO:4);

5 SKRSCSVPEGMVCK (SEQ ID NO:5);

LRWRCQRR (SEQ ID NO:6); and,

ISECKCSC (SEQ ID NO:7),

the peptide being substantially purified or having one or more therapeutic adjuvants therewith.

5. The composition as in claim 4 wherein the therapeutic adjuvant includes a neurotrophic factor.

6. The composition as in claim 4 including a physiologically acceptable solvent in which the peptide is dissolved.

7. A nucleotide sequence coding for one or more of the amino acid sequences

QMWLWSQTFCPVLY (SEQ ID NO:3);

RFWPRYVKVGSC (SEQ ID NO:4);

5 SKRSCSVPEGMVCK (SEQ ID NO:5);  
LRWRCQRR (SEQ ID NO:6); and,  
ISECKCSC (SEQ ID NO:7).

8. The nucleotide sequence as in claim 7 wherein the sequence is sufficient in size to be capable of hybridizing with endogenous mRNA for a dorsal growth inducing protein in a vertebrate.

9. The nucleotide sequence as in claim 7 wherein the amino acid sequence comprises a polypeptide having physiological activity in vertebrates.

10. The nucleotide sequence as in claim 9 wherein the physiological activity includes induction of dorsal development.

11. The nucleotide sequence as in claim 9 wherein the polypeptide is soluble in physiological solution.

12. The nucleotide sequence as in claim 9 wherein the polypeptide is comprised of at least one moiety having a molecular weight of about 25 to about 30 Kdaltons on SDS PAGE.

13. An oligonucleotide construct comprising a sequence coding for a polypeptide and an expression vector operatively linked therewith, the polypeptide being soluble when expressed in a vertebrate and being  
5 capable of inducing dorsal development in a vertebrate.

14. The construct as in claim 13 wherein the expression vector is a mammalian or viral expression vector.

15. A diagnostic or therapeutic agent comprising a nucleotide sequence of at least about fifteen DNA or RNA bases analogous to all or a portion of SEQ ID NO:1 or SEQ ID NO:2.

16. The agent as in claim 15 wherein the sequence of DNA bases or RNA bases are in the antisense direction with respect to SEQ ID NO:1 or SEQ ID NO:2.

17. A complex comprising a ligand characterized by one or more of the following sequences:

QMWLWSQTFCPVLY (SEQ ID NO:3);

RFWPRYVKVGSC (SEQ ID NO:4);

5 SKRSCSVPEGMVCK (SEQ ID NO:5);

LRWRCQRR (SEQ ID NO:6); and,

ISECKCSC (SEQ ID NO:7),

the ligand being bound to a protein.

18. The complex as in claim 17 wherein the bound protein is an antibody.

19. The complex as in claim 17 wherein the ligand has dorsal growth inducing activity and the bound protein is a receptor therefor.

20. An isolated nucleic acid that hybridizes to SEQ ID NO:1 or SEQ ID NO:2 under stringent conditions.

21. A method for identifying molecules related to SEQ ID NO:1 or SEQ ID NO:2 comprising:

screening a nucleic acid library for clones hybridizing to portions of SEQ ID NO:1 or SEQ ID NO:2;

5 isolating and propagating clones which hybridize to SEQ ID NO:1 or SEQ ID NO:2; and,

determining the sequence of said clones.

22. The method as in claim 21 wherein portions of SEQ ID NO:1 or SEQ ID NO:2 for which hybridizing clones are screened, isolated, and propagated code for one or more of SEQ ID NOS:3-7.

23. The method as in claim 21 wherein the screening includes use of one or more primers for amplification.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/08325

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12P 21/06; A61K 37/00, 37/02, 37/24

US CL : 435/69.1; 514/12, 13, 14, 21; 530/326, 327, 399

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1; 514/12, 13, 14, 21; 530/326, 327, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

IntelliGenetics, INDEX MEDICUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Development, Volume 111, issued April 1991, Christian et al, " <u>Xwnt-8</u> , a <u>Xenopus Wnt-1/int-1</u> -related gene responsive to mesoderm-including growth factors, may play a role in ventral mesodermal patterning during embryogenesis", pages 1045-1055, see entire document.	1-23
Y	Science, Volume 249, issued 03 August 1990, Sokol et al, "A Mouse Macrophage Factor Induces Head Structures and Organizes a Body Axis in <u>Xenopus</u> ", pages 561-564, see entire document.	1-23
Y	Oncogene, Volume 6, issued February 1991, Chen et al, "The proto-oncogene c-ros codes for a transmembrane tyrosine protein kinase sharing sequence and structural homology with <u>sevenless</u> protein of <u>Drosophila melanogaster</u> ", pages 257-264, see entire document.	1-23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

•	Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A	document defining the general state of the art which is not considered to be part of particular relevance		
*E	earlier document published on or after the international filing date	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O	document referring to an oral disclosure, use, exhibition or other means		
*P	document published prior to the international filing date but later than the priority date claimed	*&	document member of the same patent family

Date of the actual completion of the international search

10 DECEMBER 1993

Date of mailing of the international search report

21 DEC 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

LAURIE SCHEINER

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/08325

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 86/06096 (GINSBURG ET AL) 23 October 1986, see entire document.	1-23

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/08325

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
(Telephone Practice)

- I. Claims 1-6, drawn to polypeptides, classified in Class 530, subclass 327.
- II. Claims 7-16 and 20-23, drawn to DNA and methods of use, classified in Class 536, subclass 32.1.
- III. Claims 17-19, drawn to a complex comprising a ligand bound to a protein, classified in Class 530, subclass 350.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**